STUDII ŞI SINTEZE

ANGIOGENESIS OF ATHEROSCLEROTIC PLAQUES IN PATIENTS WITH METABOLIC SYNDROME

In memoriam V. Anestiaids

A. Munteanu[1]

post-graduate student, assistant of Pathological Anatomy Department, State University of Medicine and Pharmacy „Nicolae Testemitanu", Chisinau, Republic of Moldova

e-mail: amannberg@yandex.ru, tel. mob. +37369315977

Summary

This article is the result of our long-term studies in the area of atherogenesis and continuing to date search for answers to unresolved issues, in cooperation with the Angiogenesis Research Center, the Victor Babes University of Medicine and Pharmacy (Timisoara, Romania). The results of these studies were presented at many national and international symposia and congresses. Numerous studies have demonstrated that endothelial damage is a precursory symptom of atherosclerosis, which leads to an increase of vascular permeability, activation of mast cells and migration of leukocytes, lymphocytes, macrophages, adhesion of platelets, proliferation of vascular smooth muscle cells and eventual vasospasm and pro-inflammatory condition. All of the above listed components can be rightfully considered active pathogenetic participants in atherosclerosis and a result of aggregation of all risk factors that accompany a wide variety of cardiovascular diseases, such as coronary heart disease, hypertension, diabetes, dyslipidemia etc. The influx of monocytes and mast cells during the early stages of atherosclerosis leads to the most pronounced manifestations of vascular inflammation, especially in patients with metabolic disorders [1]. Angiogenesis is a very important pathogenetic element of atherosclerosis in stages of complicated plaques, along with mast cells and macrophages. CD-105 is a sensitive marker of newly formed endothelial cells, an effective index of activation and proliferation of microvessels, not only in aggressive forms of cancer, but also in atherosclerotic plaques of the affected vessels. The plaque neovascularization process often begins in intima, progresses and leads to further destabilization of atherosclerotic plaques (intramural hemorrhage, ruptures etc.). Also, anti-MCT (mast cell tryptase) and CD-68 demonstrate clearly the important pathogenetic stages and patterns of atherosclerosis development and its complications in patients with metabolic disorders. In our study, we analyzed the histotopographic distribution of newly formed blood vessels as a feature of angiogenesis, the extent of mast cell degranulation, the expression of macrophages in different types of plaques, as well as various arterial vessels in patients with atherosclerosis and metabolic syndrome, complicated by atherosclerosis. We have tried to analyze the importance of mast cells and macrophages, the patterns of development of atherosclerosis stages, along with diagnostic and prognostic features. The study included 34 patients, who died of atherosclerosis (no. =17) and atherosclerotic complications of metabolic syndrome (no. =17). Fragments of their cerebral (middle cerebral arteries), carotid, coronary arteries, aorta (thoracic and abdominal segments), renal, iliac and vertebral arteries were collected for research at autopsy. The fragments were processed using standard techniques. The type definition of plaques was based on morphological classification, as well as on macroscopic and histological images of hematoxylin-eosin stained sections and on histochemical methods – silver and orcein impregnation. To determine the expression of mast cells in the affected vessels, we have used anti-MCT immunohistochemical stain. Macrophages were identified using the CD-68 specific marker and the newly formed vessels – respectively, by using CD-105 (Endoglin), which is specific. The evaluation of the results was based on determining the density and intensity of the final reaction, reflected in the quantitative ratio of different zones of atheromatous plaques. Positively stained mast cells, macrophages and newly formed vessels were found in many types of atherosclerotic plaques, especially in adventitia and in the immediate vicinity of plaques and in subendothelial layers. We found a statistical correlation between the plaque type and clinical data. The immunohistochemical method is effective for determining mast cells, macrophages, and newly formed vessels of atherosclerotic plaques, directly reflecting many important pathogenetic elements of atherogenesis in patients with metabolic syndrome.

Key words: Atherosclerosis, metabolic syndrome, angiogenesis, mast cell, macrophage, stability of atherosclerotic plaque, acute cardiovascular syndromes.

Rezumat. Angiogeneza plăcilor aterosclerotice la pacienţii cu sindrom metabolic

Acest articol este rezultatul studiilor îndelungate în domeniul aterogenezei şi continuă în prezent pentru soluţionarea răspunsurilor multiplelor probleme nerezolvate, în colaborare cu Centrul de Cercetare în Angiogeneza, Universitatea de Medicina şi Farmacie „Victor Babeş" (Timişoara, România). Rezultatele acestor studii au fost prezentate la

numeroase simpozioane şi congrese naţionale şi internaţionale. Numeroase studii au demonstrat că disfuncţia endotelială este un semn precoce al aterosclerozei, ceea ce favorizează creştere a permeabilităţii vasculare, activarea mastocitelor şi migrarea leucocitelor, limfocitelor, macrofagelor, adeziunea plachetară, proliferarea celulelor musculare netede vasculare şi eventual vasospasmul, ce în ansamblu determină un statut pro-inflamator. Toate componentele mai sus menţionate pot fi considerate participanţi patogenetici activi în ateroscleroză şi un rezultat de agregare a tuturor factorilor de risc care însoţesc o varietate mare de boli cardiovasculare acute, cum ar fi boala coronariană, hipertensiunea, diabetul zaharat, dislipidemiile etc. Afluxul de monocite şi mastocite la etapele precoce ale aterosclerozei, determină manifestările mai pronunţate ale inflamaţiei vasculare, în special, la pacienţii cu tulburări metabolice [1]. Angiogeneza este un element patogenetic important al aterosclerozei la etape de plăci complicate, împreună cu mastocite şi macrofage. CD - 105 este un marker sensibil al celulelor endoteliale noiformate, un indice efectiv al activării şi proliferării microvaselor, nu numai în forme agresive de cancer, dar şi în plăcile aterosclerotice ale vaselor afectate. Procesul de neovascularizaţie a plăcilor ateromatoase, adesea începe de la intimă, progresează şi favorizează destabilizarea în continuare a plăcilor aterosclerotice (rupturi, hemoragii etc.). De asemenea, anti-MCT (triptaza mastocitară) şi CD - 68 demonstrează în mod clar etapele importante patogenetice şi modele de dezvoltare a aterosclerozei şi a complicaţiilor sale, la pacienţii cu sindromul metabolic. În studiul de faţă, am analizat distribuţia histotopografică a vaselor noiformate ca o caracteristică a angiogenezei, gradul de degranulare a mastocitelor, expresia macrofagelor în diferite tipuri de plăci, în diferite vase arteriale la pacienţi cu ateroscleroză şi sindromul metabolic complicat cu ateroscleroza. Noi am încercat să analizăm importanţa mastocitelor şi macrofagelor, etapelor de dezvoltare a aterosclerozei, de asemenea, criteriile de diagnostic şi prognostic. Studiul nostru a inclus 34 de pacienţi, care au decedat de ateroscleroza (nr. = 17) şi complicaţiile aterosclerotice ale sindromului metabolic (nr. = 17). Fragmente de aa. cerebrale (arterele cerebrale medii), aa. carotide, arterele coronare, aorte (toracice şi segmente abdominale), aa. renale, aa. iliace şi arterele vertebrale au fost prelevate pentru cercetare în timpul necropsiilor. Fragmentele au fost prelucrate folosind tehnicile histologice uzuale. Determinarea tipurilor de placi a fost bazată pe clasificarea morfologică, macroscopică şi histopatologică ale secţiunilor colorate Hematoxilină-Eozină, histochimice - Impregnare Argentică şi Orceină. Pentru a determina expresia celulelor mastocitare în vasele afectate, am folosit anti-MCT. Macrofagele au fost identificate folosind CD-68 markerul specific şi pentru vasele noiformate - respectiv, prin aplicarea CD-105 (endoglin), care este specific. Evaluarea rezultatelor sa bazat pe determinarea densităţii şi intensităţii reacţiei finale, reflectate în raportul cantitativ al diferitelor zone ale plăcilor ateromatoase. Mastocitele pozitiv colorate, macrofage şi vasele noiformate s-au găsit în mai multe tipuri de plăci aterosclerotice, în special în adventice şi în imediată apropiere a plăcilor şi în straturile subendoteliale. Am găsit o corelaţie statistică între tipul de placă şi datele clinice. Metoda imunohistochimică este eficientă pentru determinarea mastocitelor, macrofagelor şi vaselelor noiformate ale plăcilor aterosclerotice, în mod direct reflectând multe elemente patogenice importante ale aterogenezei la pacienţii cu sindrom metabolic.

Cuvinte-cheie: ateroscleroza, sindrom metabolic, angiogeneza, mastocit, macrofag, stabilitatea plăcilor aterosclerotice, sindroamele cardiovasculare acute.

Резюме. Ангиогенез атеросклеротических бляшек у пациентов с метаболическим синдромом

Данная статья является результатом наших длительных исследований в области атерогенеза и продолжающихся, на сегодняшний день, поисков ответов на неразрешённые вопросы совместно с Научным Центром по Изучению Ангиогенеза, Университета Медицины и Фармации им. Виктор Бабеш (Тимишоара, Румыния), результаты которых были представлены на многих национальных, международных симпозиумах и конгрессах. Многочисленные исследования демонстрируют, что повреждение эндотелия является ранним предвестником атеросклероза, которая приводит к повышению проницаемости сосудов, активации мастоцитов, миграции лейкоцитов, лимфоцитов, макрофагов, адгезии тромбоцитов, пролиферации гладкомышечных клеток сосудов и в конечном счете вызывая спазм сосудов и провоспалительный статус. Все выше перечисленные компоненты можно полноправно считать активными патогенетическими участниками атеросклероза и как результат агрегации всех факторов риска сопровождающие широкий спектр сердечно-сосудистых заболеваний, таких как ишемическая болезнь сердца, гипертония, сахарный диабет, дислипидемии и т.д. Приток мастоцитов и моноцитов на ранних стадиях атеросклеротического процесса приводит к наиболее выраженным проявлениям сосудистого воспаления, особенно у пациентов с метаболическими расстройствами [1]. Важнейшим патогенетическим звеном атеросклероза, в стадиях осложнённых бляшек, является ангиогенез, на ряду с мастоцитами и макрофагами. CD-105 является чувствительным маркером новообразованых эндотелиальных клеток, эффективным показателем активации и пролиферации микрососудов, не только в агрессивных формах злокачественных опухолей, но и в атеросклеротических бляшках поражённых сосудов. Процесс неоваскуляризации бляшек, зачастую начинается с интимы, прогрессирует и приводит к дальнейшей дестабилизации атеросклеротических бляшек (интрамуральные кровоизлияния, разрывы и др.). Также анти-MCT (mast cell tryptase) и CD-68 наглядно демонстрируют важные патогенетические этапы и закономерности развития атеросклероза и его осложнений у пациентов с метаболическими нарушениями. В нашем исследовании мы проанализировали гистотопографическое распределение новообразованных сосудов как особенности ангиогенеза, степень дегрануляции мастоцитов, экспрессию макрофагов в различных типах бляшек, а также разных артериальных сосудов у пациентов с

атеросклерозом и метаболическим синдромом осложненным атеросклерозом. Мы попробовали проанализировать значение мастоцитов и макрофагов, закономерностей развития стадий атеросклероза, наряду с диагностическими и прогностическими особенностями. В исследование было включено 34 пациента, умерших от атеросклероза (n = 17) и атеросклеротических осложнений метаболического синдрома (n = 17), у которых при вскрытии были взяты для исследований фрагменты мозговых (средние мозговые артерии), сонных артерий, коронарных артерий, аорты (грудной и брюшной отделов) почечных, подвздошных и позвоночных артерий. Фрагменты были обработаны с помощью стандартных методик. Определение типа бляшек основывалось на морфологической классификации, а также на основании макроскопической и гистологической картины окрашенных Г.-Э. срезов и гистохимических методов – импрегнирование серебром и орсеином. Для определения экспрессии мастоцитов в пораженных сосудах мы использовали иммуногистохимическую окраску анти-МСТ (mast cell tryptase). Макрофаги были выявлены применяя специфический маркер CD-68, а новообразованные сосуды – соответственно, CD-105 (Эндоглин), являющийся специфичным. Оценка результатов была основана на определении плотности и интенсивности окончательной реакции, отраженной в количественном соотношении различных зон атероматозных бляшек. Положительно окрашенные мастоциты, макрофаги и новообразованные сосуды выявлены во многих типах атеросклеротических бляшек, и особенно в адвентиции, так же обнаружены в непосредственной близости от бляшек и в субэндотелиальных слоях. Мы обнаружили статистическую корреляцию между типа бляшки и клинических данных. Иммуногистохимический метод эффективен для определения мастоцитов, макрофагов и новообразованых сосудов атеросклеротических бляшек и непосредственно отражает многие важные патогенетические звенья атерогенеза у больных с метаболическим синдромом.

Ключевые слова: Атеросклероз, метаболический синдром, ангиогенез, мастоцит, макрофаг, стабильность атеросклеротической бляшки, острые сердечно-сосудистые синдромы

Relevance of the problem

Cardiovascular diseases constitute the most common cause of premature death in industrialized countries, accounting for 4.35 million deaths annually in Europe, and 35% - in the UK [2]. Despite the significant progress made in clinical methods of diagnosis and symptomatic treatment of cardiovascular diseases and metabolic syndrome, atherosclerosis is still a leading cause of morbidity and mortality not only in Moldova, but also worldwide. The leading role of inflammation in atherogenesis acquired wide recognition in the scientific community and forced us to rethink our ideas about the stages of formation of atherosclerotic plaques.

Atherosclerosis is directly related to symptoms of cardiovascular diseases and strokes, major consequences of which are death and disability. Atherosclerosis is a polyetiological disease with numerous risk factors, including smoking, alcohol abuse, hypertension, diabetes, dyslipidemia, and infection. All these factors involve complex interactions between various components - inflammation, lipid metabolism, blood coagulation system, hypoxia, apoptosis and immune response. The instability of the atherosclerotic plaque is a relatively independent risk factor for ischemic stroke [4,5,6,7]. In the absence of atherosclerosis, normal vessel walls have their microcirculatory bed limited by an adventitious membrane [8]. Intima of the newly formed vessels, associated to atherosclerotic plaques, was first studied in 1876 by Koester [9].

In atherosclerotic plaques, angiogenesis allows the formation of new microvessels, in order to maintain the necessary level of oxygen and nutrients in the vascular wall [7].

The growth of newly formed vessels occurs in areas of atherosclerotic lesions that undergo constant changes, renovations and are prone to rupture. Some studies show that the formation of new blood vessels contributes to growth of atherosclerotic lesions and is a key factor leading to plaque rupture and destabilization [10,11]. Some of the newly formed blood vessels are immature and similar to those observed in neovascularization of solid tumors, and, therefore, they may contribute to the development of hemorrhage in plaques and subsequent instability [6.8.12]. CD-105 is a homodimeric, integral membrane glycoprotein, consisting of 90-95 kDa subunits, with disulfide links [13]. It is a component of transforming growth factor beta, TGF-β, receptor complex. CD105 appears in angiogenic endothelial cells [14,15].

CD-105 is a sensitive marker for identification of newly formed blood vessels and, in tumors – their growth and outcome prediction [16,17].

CD-105 is a specific and sensitive marker for the evaluation of newly formed vessels in atherosclerotic plaques [18,19]. Determining the level of circulating soluble CD-105 sensitive antigens can determine exactly the presence of unstable plaques or their ruptures [20].

The three main types of cells - mast cells, macrophages and T-lymphocytes - usually constitute the inflammatory cell infiltrates of atherosclerotic plaques and intercellular substance [21,22].

The immune response is composed of three types of activated inflammatory cells, which interact simul-

taneously (V. Anestiadi, V. Nagornev, E. Zota) [23]. Mast cells are formed from stem cells of the bone marrow and circulate freely in the peripheral blood. The granules of mast cells contain a number of mediators, such as neutral protease, tryptase, chymase, cathepsin G, histamine, heparin, a large number of cytokines and chemokines, tumor necrosis factor (TNF-α), interleukins (IL), vascular endothelial growth factors (VEGF) and basic fibroblast growth factor (bFGF) [24]. The infiltration by inflammatory cells of the vessel wall, in particular the intima, has an important role in the pathogenesis of atherosclerosis development and, probably, is the main cause of acute cardiovascular syndromes (e.g. myocardial infarction and stroke). Atherosclerosis is currently regarded as a complex metabolic disorder with progression of chronic inflammatory processes. An important peculiarity is the ability of mast cells to release their cytoplasmic content (granules) and become active in the extracellular space. Activated mast cells secrete large amounts of chemotactic molecules, activators of inflammatory reactions. In addition, these same mast cells bind nonspecifically to the low density lipoproteins (LDL), which can be phagocytosed by macrophages and then form foam cells, the main cellular component of atherosclerotic lesions (V. Anestiadi, V. Nagornev, E. Zota) [25].

Mast cells have different functions that can modulate the atherogenesis in natural conditions. Mast cells are crucial in the development of atherosclerotic plaque. The participation of mast cells in atherogenesis allows us to really interpret the phenomena associated with clinical manifestations, early diagnostic prospects, to identify therapeutic targets of treatment and individual prognosis in patients with metabolic syndrome complicated by atherosclerosis. The manifestations of different stages of atherosclerotic plaques, in different types of arterial vessels, are described incompletely and the expression of markers specific to mast cells has not been fully studied to date.

We have noted the statistical correlation between the type of plaque and the clinical data. The immunohistochemical method is effective for determining mast cells of atherosclerotic plaque and directly reflects the enzymatic activity of mast cell proteases, some important pathogenetic links of atherogenesis in patients with metabolic syndrome. The obtained data can serve as a substrate for further research to identify potentially valuable and new methods for early lifetime diagnostics and therapeutic purposes.

Research objective

Microscopic analysis of histotopographical distribution of newly formed blood vessels, the extent of mast cell degranulation, macrophage expression in different stages of plaque development, as well as various types of arterial vessels in patients with arteriosclerosis and metabolic syndrome complicated by atherosclerosis.

Also, we have analyzed the importance of mast cells and macrophages, the patterns of development of atherosclerosis stages, statistical correlations along with diagnostic and prognostic features.

Material and methods

The evaluation of results was based on the determination of density distribution of staining and intensity of the final reaction, reflected in the quantitative ratio of the different zones of atheromatous plaques. Positively stained mast cells were found in many types of atherosclerotic plaques, especially in adventitia and in the immediate vicinity of plaques and in subendothelial layers.

34 patients with atherosclerosis and metabolic syndrome were investigated. Representatives of all age groups were among them, but the degree of affection increased after the age of 40. The age ranged from 44 to 83 years (the average age was 62.8 years). There were 14 women (41, 2%) and 20 men (the average age constituted 58, 8%), who died of atherosclerosis (no. =17) and atherosclerotic complications of the metabolic syndrome (no. =17). Fragments of their cerebral (middle cerebral arteries), carotid, coronary arteries, aorta (thoracic and abdominal segments), renal, iliac and vertebral arteries were collected for research at autopsy.

The vessel fragments were processed according to standard procedures (fixed in 10% buffered formalin solution, enclosed in paraffin blocks and 5.4 micrometer thick sections were obtained). The definition of the plaque type and stage was based on the AHA (*American Heart Association, 1995*) morphological classification [24], taking into account the macroscopic, histological image of the hematoxylin-eosin stained sections and histochemical methods - silver and orcein impregnation.

Additional sections of paraffin blocks were processed immunohistochemically. The sections were deparaffinized, hydrated, and then, the reaction to antigen detection in the PT Link module (DakoCytomation, Denmark) was carried out. The next stage was the incubation of primary antibodies, using NovoLink Max Polymer Detection System, and, to visualize the final reaction, we used 3.3 of diaminobenzidine dihydrochloride, as a chromogen with brown staining. A more detailed description of the immunohistochemical procedures is presented in Table 1.

CD-105 (Endoglin) was used to determine the immunohistochemical expression of the endothelium

Table 1

IHC detection system of mast cells, macrophages and newly formed blood vessels

Marker	*Manufacturer*	*Clone*	*Concentration*	*Detection system*	*Antigen retrieval*	*Incubation of primary antibody*
CD105 Endoglin	*Dako Glostrup, Denmark*	*Monoclonal mouse antihuman, clone SN6h*	*1: 10*	*NovoLink Max Polymer Detection System*	*Proteinase K, 10 minutes*	*30 minutes, indoor temperature*
anti-MCT (mast cell tryptase)	*Neo-Markers Fremont, CA*	*Mouse Monoclonal Antibody, clone AA1)*	*RTU (concentration prepared for use)*	*NovoLink Max Polymer Detection System*	*Microwaves, 30 minutes, pH 6*	*30 minutes, indoor temperature*
CD68	*Dako Glostrup, Denmark*	*Monoclonal mouse antihuman, QBEnd 10*	*1:25*	*NovoLink Max Polymer Detection System*	*Microwaves, 30 minutes, pH 6*	*30 minutes, indoor temperature*

of newly formed blood vessels, as well as anti-MCT (mast cell tryptase) for mast cells and CD-68, respectively, for macrophages. All the immunohistochemical procedures were performed using the DakoAutostainer Plus (DakoCytomation, Denmark) automated system.

Quantitative analysis: The hot-spot method [26] is the most effective and frequently used method for quantitative determination of histological structures. Using an optical microscope, the areas of interest are studied at 200× magnification, which corresponds to the surface of 0.74 mm^2. The method consists in the study of three fields with the highest density of histological structures (capillaries, cells), and then recounting and calculation of the arithmetic mean.

In the statistical analysis, we used the commercial software SPSS 19.0 and Microsoft Office 2013 (Microsoft Excel Worksheet), as well as x^2 and Student tests, $p<0.05$.

To investigate the histological specimens and obtain images, we used the microscopes Nikon Eclipse E 600 (Nikon, Japan), Nikon Labothot-2 and Carl Zeiss Axiolab. All the immunohistochemical investigations were carried out at the Angiogenesis Research Center (Timisoara, Romania).

All clinical data and results of lifetime laboratory tests were used from patients' medical records.

Table 2

Biochemical laboratory data of the patients included in the study

	Atherosclerosis	*Metabolic syndrome*
Age (average)	*63,4*	*62,2*
Gender (♂/♀)	*11/6*	*9/8*
Blood glucose (mmol/l)	*4,74*	*16,2*
Total cholesterol (mmol/l)	*6,90*	*6,01*
Triglycerides (mmol/l)	*0,86*	*1,58*
HDL (mmol/l)	*1,003*	*1,023*
LDL (mmol/l)	*1,99*	*3,29*
Leukocytes (×10^9/l)	*8,42*	*8,75*
Lymphocytes (%)	*24,5*	*26,7*
Monocytes (%)	*7,57*	*6,21*
Prothrombin (%)	*73,6*	*78,6*
Fibrinogen (g/l)	*3,3*	*5,39*
ESR (mm/hour)	*17,5*	*25,5*

Estimation of results

Initially, to identify the correlation between the expression of mast cells, macrophages and newly formed blood vessels, their histotopographic localization and histopathological types of atherosclerotic plaques, we used the conventional histopathological classification, in accordance with WHO recommendations.

In order to evaluate the intensity of the reactions, we used the principle based on the number of positively stained cells and blood vessels, and namely: negative (denoted by 0), slightly positive with less than 10% of stained cells (1+), moderately positive with 11-50% of stained cells (2++), and strongly positive with more than 50% of stained cells (3+++). The intensity of the final reaction was not taken into account, because all stained cells showed an intense reaction, even if they were few and isolated. The number of mast cells was assessed by two observers simultaneously, using the arithmetic mean of positive cells in the three respective fields of view (provided that they were present).

Statistical analysis: For statistical analysis, the commercial software Microsoft Office 2013 (*Microsoft Excel Worksheet*) and Spearman type correlation tests were used. The statistically significant $p<0,05$ correlation was taken into account. Correlations were found between the expression of mast cells, macrophages and newly formed vessels, their histotopographic distribution in the atherosclerotic plaques of various types of vessels and clinical factors considering the laboratory data.

Table 3

Quantitative distribution of histopathological types of atherosclerotic plaques

	Type of lesion / artery	Cerebral arteries	Carotid arteries	Aorta	Coronary arteries	Renal arteries	Iliac arteries	Vertebral arteries	Total of plaque types
Group 1 (A)	**IL**	*5*	*2*	*0*	*5*	*5*	*2*	*0*	**19**
	FP	*9*	*7*	*7*	*7*	*7*	*10*	*4*	**51**
	CFP	*3*	*8*	*10*	*5*	*5*	*5*	*1*	**36**
Group 2 (MS)	**IL**	*2*	*0*	*0*	*2*	*2*	*0*	*0*	**6**
	FP	*10*	*10*	*11*	*13*	*13*	*10*	*12*	**79**
	CFP	*5*	*7*	*6*	*2*	*2*	*7*	*0*	**29**
Total	**IL**	**7**	**2**	**0**	**7**	**7**	**2**	**0**	**25**
	FP	**19**	**17**	**18**	**20**	**20**	**20**	**16**	**130**
	CFP	**8**	**15**	**16**	**7**	**7**	**12**	**1**	**66**

IL *– intermediate lesion,* ***FP*** *– fibrous plaque,* ***CFP*** *– calcified fibrous plaque.*

Results and discussion

The histopathological analysis revealed the existence of three major (conditional) types of atherosclerotic lesions in the two study groups: intermediate lesion (IL), fibrous (formed) plaque (FP), calcified fibrous plaque (and/or complicated) (CFP) in all studied vessels, which, in turn, were stained with hematoxylin-eosin, orcein and impregnated with silver. The histopathological evaluation with determination of microscopic stage and type of atherosclerotic plaques is shown in Table 3.

The final, positive, immunohistochemical reaction to anti-MCT manifested itself as staining in the form of granular cytoplasmic pattern, limited by mast cells (some of them with degranulation). The ratio of expression of anti-MCT and macrophages, with histotopographic distribution at plaque level, is shown in Figures 1 and 2.

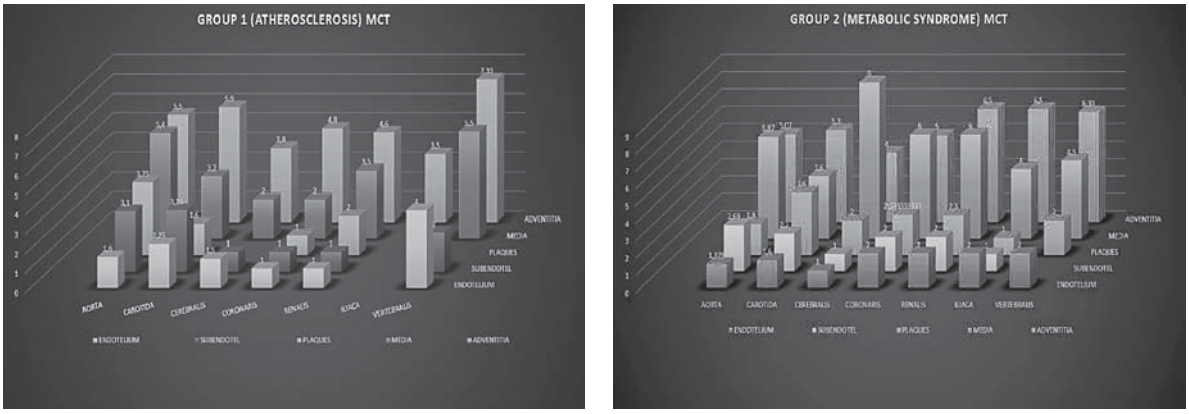


Figure 1. *Histotopographic expression of mast cells in atherosclerotic plaques of various types of vessels*.

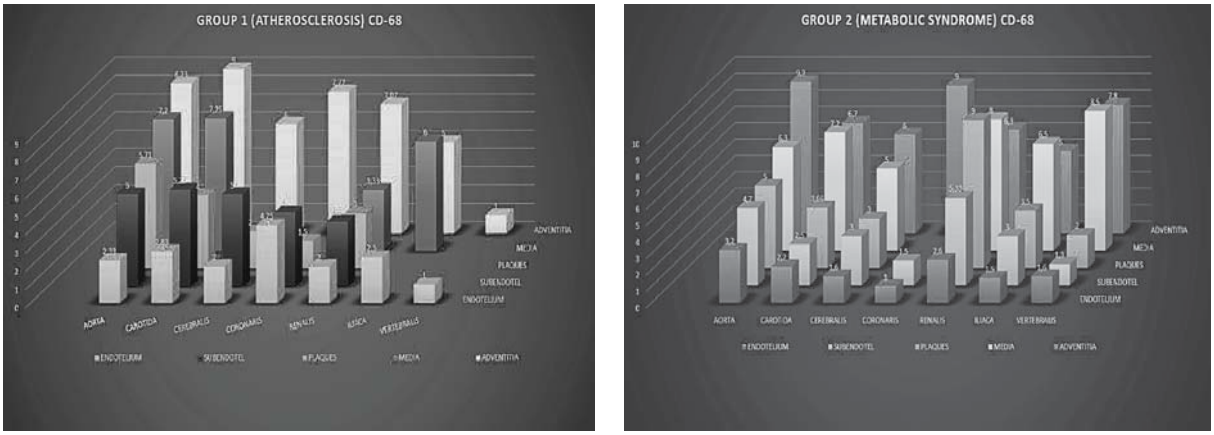
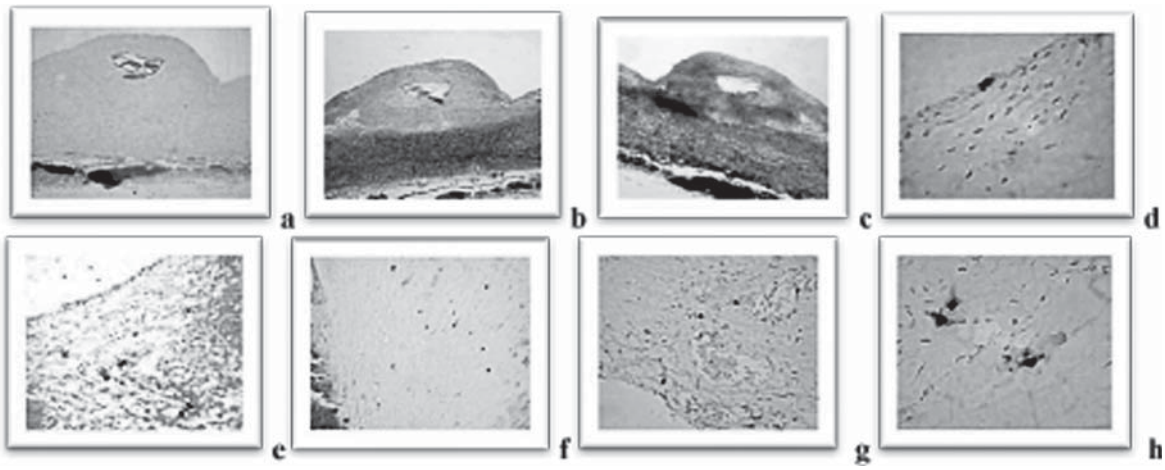
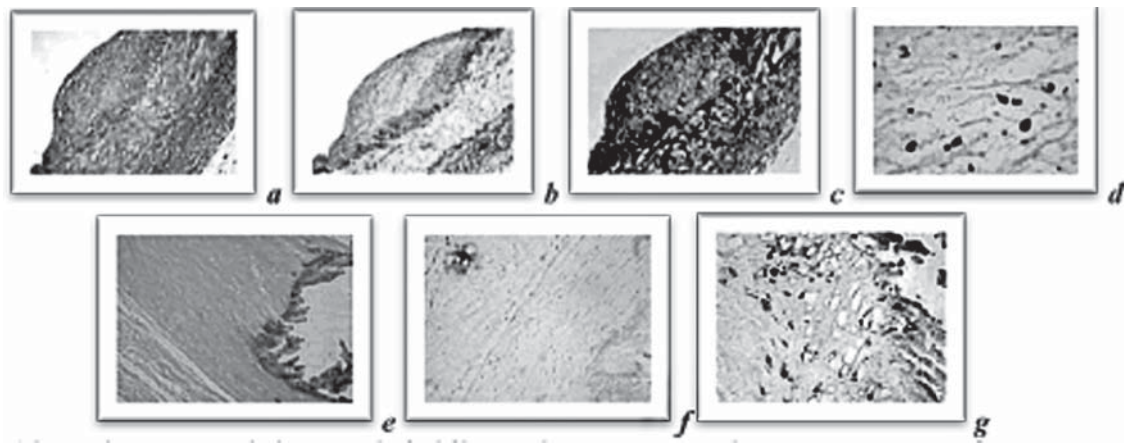


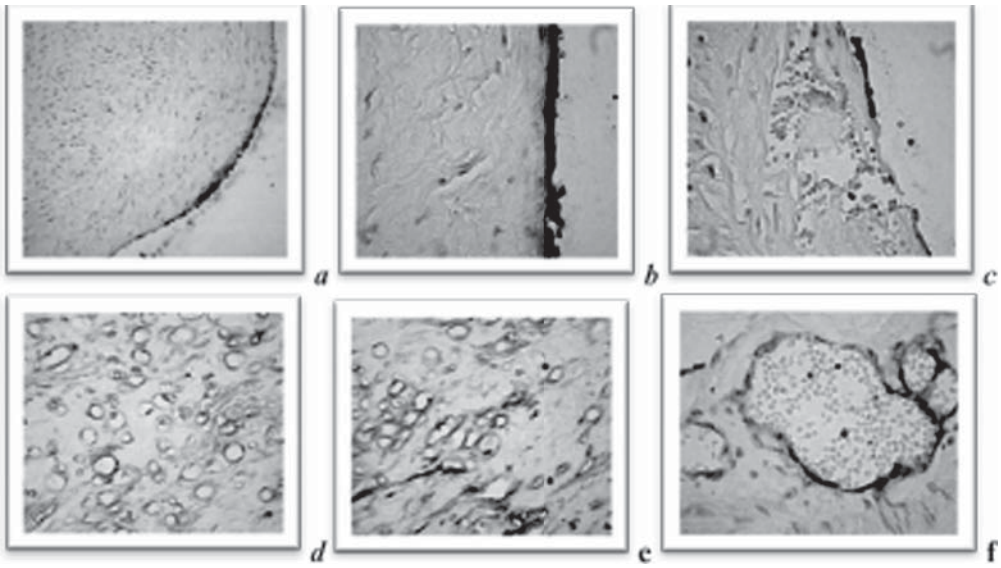
Figure 2. *Histotopographic expression of macrophages in atherosclerotic plaques of various types of vessels*.



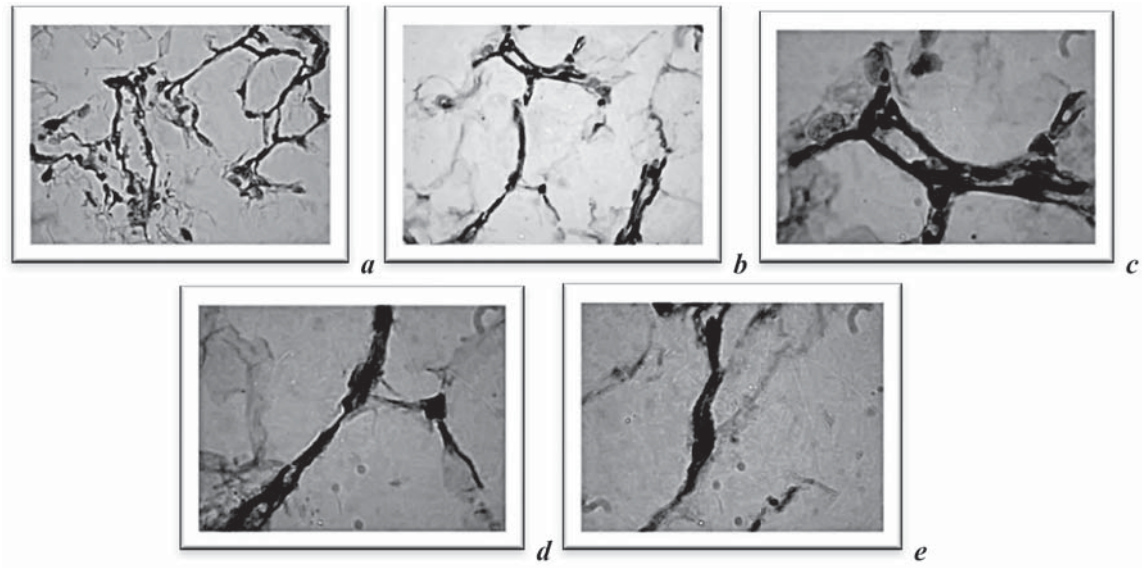
Picture 1. *Aortic atherosclerotic plaque (calcified fibrous plaque). Hematoxylin-eosin staining (a); Orcein staining (b); Staining - silver impregnation (c); positively stained (anti-MCT) mast cells in endothelium (d); positively stained (anti-MCT) mast cells in subendothelium (e); positively stained (anti-MCT) mast cells in close proximity to the area of calcification (f); positively stained (anti-MCT) mast cells in proximity to adventitia in plaque site (g) and aloof from it (h)). Original magnification 100x and 400x.*



Picture 2. *Atherosclerotic carotid plaque (calcified fibrous plaque). Hematoxylin-eosin staining (a); Orcein staining (b); Staining - silver impregnation (c); positively stained (anti-MCT) mast cells in fibrous plaque (d); calcified focus in the carotid artery (e); degranulated, positively stained mast cell near calcification focus (f); positively stained (anti-MCT) mast cells in close proximity to the area of calcification (g). Original magnification 100x and 400x.*



Picture 3. Complicated atherosclerotic carotid plaque (fibrous plaque) – moderately and intensively positive CD-105-stained endothelium (a, b, c,); Complicated aortic atherosclerotic plaque (fibrous plaque) – many newly formed blood vessels and intensively positive CD-105-stained endothelium (d, e, f). CD-105 staining. Original magnification 100x and 400x.



Picture 4. *Complicated atherosclerotic carotid plaque (calcified fibrous plaque) – moderately, positively CD-105-stained endothelial cells with a tendency to form plexus and capillaries (a, b, c, d, e). CD-105 staining. Original magnification 400x and 1000x.*

Conclusion

In this study, we have shown the existence of differential expression of anti-MCT, CD-68 and CD-105 (Endoglin) in different types of atherosclerotic lesions and different types of vessels, in association with histotopographic distribution.

The data obtained confirm the theory of evolution and the pathogenetic mechanisms of atherogenesis. Despite these assertions, today there are still many unresolved scientific and clinical problems.

For comparison, one can cite as an example the angiogenesis of tumor processes, in which mast cells and macrophages, likewise in the formation of atherosclerotic plaques, are involved in neovascularization (only discussing general mechanisms, functions and pathophysiological components).

Anti-MCT and CD-68 are selective markers for mast cells, macrophages, which are important components of the immune processes in the initiation, proliferation and differentiation of cells in atherosclerotic lesions. In addition to T-lymphocytes and macrophages, other immune effector cells are also involved in atherosclerotic lesions, while lymphocytes, macrophages prevail over mast cells, which perform an important function in the development of atherosclerotic plaque in different vessels, explained by production of large quantities of proteases, including those produced by macrophages, with their accumulation in the necrotic nucleus of the plaque.

The factors produced by mast cells and macrophages may contribute to the destruction of the intercellular matrix and cause an additional modification of LDL. Most of the studied vessels were positively anti-MCT- and CD-68- stained in the endothelium, atherosclerotic plaque, tunica media and adventitia, as well as in vasa vasorum.

Obviously, the endothelial cells (EC), mast cells, macrophages and lymphocytes are effector cells, involved in atherogenesis with development of atherosclerotic plaques in patients with atherosclerosis and MS. Mast cells regulate the behavior of SMC (smooth muscle cells), most likely through their secreted mediators. The collagen fibers, produced by SMC, may prevent ruptures of atherosclerotic plaques. Nevertheless, the chymase inhibits mast cell proliferation and collagen synthesis of SMC, thereby decreasing the stability of the plaque. The action of mast cell proinflammatory cytokines, such as TNF-α, induces the expression of SMC protease. TNF-α-positive mast cells, MMP-cysteine cathepsin - positive SMC proteases, together with macrophages, suggest a regulatory role in the expression of cellular mediators, mast cell proteases in SMC activation in sites of atherosclerotic plaque rupture. Localization of mast cells and macrophages in the vessel wall, particularly perivascularly and in intima, assumes an important role in the pathogenesis of atherosclerosis and, perhaps, is the major cause of acute cardiovascular diseases (myocardial infarction and cerebral stroke, in particular).

The role of angiogenesis in the development of atherosclerosis is probably complex and depends on the stage of the pathological process. The development of microvessels in atheromatous plaques is the result of neovascularization; these newly formed capillaries are fragile and prone to rupture with hemor-

rhage. Fibrin deposits in the plaques, the formation of hemosiderin and the beginning of immune inflammation are evidence of bleeding within atheromatous lesions. The importance of angiogenesis in the destabilization and destruction of atherosclerotic plaques remains an unresolved issue, but some of the recent judgment on the underlying causes of plaque instability can lead to a new, promising interpretation of atherogenesis in general.

Patterns of development of atherosclerotic plaques (stability or instability) depend, to great extent, on the angiogenesis of the atherosclerotic process. Our results show that the comparative immunohistochemical method using vascular markers demonstrates the important pathogenetic aspects in the formation of atherosclerotic plaques. Mast cells and macrophages, as well as other immunocompetent cells, play an important role in the development of atherosclerotic plaques and, last but not least, in the angiogenesis process. The question arises whether the inhibition of angiogenesis could be a therapeutic target in atherosclerosis or how it can be used in the metabolic syndrome. Available data indicate that antiangiogenic therapy may have a potential impact on the development of neointima in atherosclerotic lesions, and side effects and exposure to harmful factors are likely to inhibit the endothelium function and regeneration. These assertions are supported by scientific data obtained by many laboratories, which demonstrate that VEGF has a protective effect on the endothelium of the arteries. Recent clinical studies of VEGF inhibitor antibodies, when using Bevacizumab (Avastin) in malignant tumors, indicate that up to 5% of all patients receiving Avastin may have an increased risk of thromboembolism, including acute stroke, myocardial infarction and deep phlebothrombosis. These data suggest that endogenous VEGF may play a certain atheroprotective role in vascularization. The plurality of VEGF biologically important functions and the integrity of vascular endothelium functions are solid arguments that currently limit any antiangiogenic approaches for the treatment of cardiovascular diseases.

CD-105 is a valuable marker of angiogenesis of atherosclerotic plaques, intimal arteries and adventitial vessels, an indicator of the degree of variation in the pathological development of atherosclerosis - the factors that may be important in introducing modern methods of research, diagnosis, treatment and prognosis of these diseases.

Bibliography

1. Ley K., Miller Y.I., Hedrick C.C. Monocyte and macrophage dynamics during atherogenesis. Arterioscler. Thromb. Vasc. Biol.: 2011; 31(7);1506–1516.

2. Peterson S., Peto V. and Rayner M.: European Cardiovascular Disease Statistics, British Heart Foundation, London. 2005.

3. Krupinski J., Font A., Luque A., Turu M., Slevin M. Angiogenesis and inflammation in carotid atherosclerosis. Front. Biosci. 2008; 13:6472-6482.

4. McCarthy M.J., Loftus I.M., Thompson M.M., Jones L., London N.J., Bell P.R., Naylor A.R., Brindle N.P. Angiogenesis and the atherosclerotic carotid plaque: an association between symptomatology and plaque morphology. J. Vasc. Surg.: 1999; 30:261-268.

5. Dunmore B.J., McCarthy M.J., Naylor A.R., Brindle N.P. Carotid plaque instability and ischemic symptoms are linked to immaturity of microvessels within plaques. J. Vasc. Surg.; 2007; 45:155-159.

6. Mofidi R., Crotty T.B., McCarthy P., Sheehan S.J., Mehigan D., Keaveny T.V. Association between plaque instability, angiogenesis and symptomatic carotid occlusive disease. Br. J. Surg. 2001; 88:945-950.

7. Mofidi R., Powell T.I., Crotty T., Mehigan D., Macerlaine D., Keaveny T.V. Angiogenesis in carotid atherosclerotic lesions is associated with timing of ischemic neurological events and presence of computed tomographic cerebral infarction in the ipsilateral cerebral hemisphere. Ann. Vasc. Surg. 2008; 22:266-272.

8. O'Brien E.R., Garvin M.R., Dev R., Stewart D.K., Hinohara T., Simpson J.B., Schwartz S.M. Angiogenesis in human coronary atherosclerotic plaques. Am. J. Pathol. 1994; 145:883-894.

9. Zhang Y., Cliff W.J., Schoefl G.I., Higgins G. Immunohistochemical study of intimal microvessels in coronary atherosclerosis. Am. J. Pathol. 1993; 143:164-172.

10. Khurana R., Simons M., Martin J.F., Zachary I.C. Role of angiogenesis in cardiovascular disease: a critical appraisal. Circulation. 2005; 112:1813-1824.

11. Tureyen K., Vemuganti R., Salamat M.S., Dempsey R.J. Increased angiogenesis and angiogenic gene expression in carotid artery plaques from symptomatic stroke patients. Neurosurgery. 2006; 58:971-977. Discussion 971-977.

12. Ribatti D., Levi-Schaffer F., Kovanen P.T. Inflammatory angiogenesis in atherogenesis a double-edged sword. Ann. Med. 2008, 40:606-621.

13. Barbara N.P., Wrana J.L., Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J. Biol. Chem. 1999; 274:584-594.

14. Duff S.E., Li C., Garland J.M., Kumar S. CD105 is important for angiogenesis: evidence and potential applications. Faseb. J. 2003; 17:984-992.

15. Guo B., Kumar S., Li C., Slevin M., Kumar P. CD105 (Endoglin), apoptosis, and stroke. Stroke. 2004; 35:94-95.

16. Dhakal H.P., Naume B., Synnestvedt M., Borgen E., Kaaresen R., Schlichting E., et al: Vascularization in primary breast carcinomas: its prognostic significance and relationship with tumor cell dissemination. Clin. Cancer. Res. 2008; 14:2341-2350.

17. Marioni G., Giacomelli L., D'Alessandro E., Staffieri C., Guzzardo V., Staffieri A., Blandamura S. Laryngeal carcinoma recurrence rate and disease-free interval are related to CD105 expression, but not to vascular endothelial growth factor 2 (Flk-1/Kdr) expression. Anticancer Res. 2008; 28:551-557.

18. Li C., Mollahan P., Baguneid M.S., McMahon R.F., Kumar P., Walker M.G., Freemont A.J., Kumar S. A comparative study of neovascularisation in atherosclerotic plaques using CD-31, CD-105 and TGF-beta1. Pathobiology. 2006; 73:192-197.

19. Middleton J., Americh L., Gayon R., Julien D., Mansat M., Mansat P., Anract P., Cantagrel A., Cattan P., Reimund J.M., et al. A comparative study of endothelial cell markers expressed in chronically inflamed human tissues: MECA-79, Duffy antigen receptor for chemokines, von Willebrand factor, CD-31, CD-34, CD-105 and CD-146. J. Pathol. 2005; 206:260-268.

20. Cui S., Lu S.Z., Chen Y.D., He G.X., Meng L.J., Liu J.P., Song Z.Y., Liu X.L., Song X.T., Ge C.J., Liu H. Relationship among soluble CD-105, hypersensitive C-reactive protein and coronary plaque morphology: an intravascular ultrasound study. Chin. Med. J. (Engl.). 2008; 121:128-132.

21. Hansson G.K. and Libby P. The immune response in atherosclerosis: a double-edged sword. Nat. Rev. Immunol.: 2006; 6: 508 – 519.

22. Hansson G.K.: Inflammation, atherosclerosis and coronary artery disease. N. Engl. J. Med.: 2005; 352, 1685 – 1695.

23. Anestiadi V., Nagornev V., Zota E. Inflammation as a cause of various forms of destruction of atherosclerotic plaques. Pathomorphosis of atherosclerosis (immunological aspects). Chisinau – Saint-Petersburg: 2008; 7, 179 – 198.

24. Kovanen P.T.: Mast cells – multipotent local effector cells in atherothrombosis. Immunol. Rev.: 2007; 217, 105 – 122.

25. Anestiadi V., Nagornev V., Zota E. Role of monocytes/macrophages in the formation and development of atherosclerotic lesions. Atherogenesis. Chisinau – Saint-Petersburg: 2001; 8.1. 158-181.

26. Van der Auwera I., Cao Y., Tille J.C., Pepper M.S., Jackson D.G., Fox S.B., Harris A.L., Dirix L.Y., Vermeulen P.B. First International consensus on the methodology of lymphangiogenesis quantification in solid human tumours. Br. J. Cancer 2006, 1-15.